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Dynamics of Introduced Populations of *Phragmidium violaceum* and Implications for Biological Control of European Blackberry in Australia▽

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***Phragmidium violaceum* causes leaf rust on the European blackberry (*Rubus fruticosus* L. aggregate). Multiple strains of this pathogen have been introduced into southern Australia for the biological control of at least 15 taxa of European blackberry, a nonindigenous, invasive plant. In climates conducive to leaf rust, the intensity of disease varies within and among infestations of the genetically variable host. Genetic markers developed from the selective amplification of microsatellite polymorphic loci were used to assess the population genetic structure and reproductive biology of *P. violaceum* within and among four geographically isolated and diseased infestations of the European blackberry in Victoria, Australia. Despite the potential for long-distance aerial dispersal of urediniospores, there was significant genetic differentiation among all populations, which was not associated with geographic separation. An assessment of multilocus linkage disequilibrium revealed temporal and geographic variation in the occurrence of random mating among the four populations. The presence of sexual spore states and the results of genetic analyses indicated that recombination, and potentially random migration and genetic drift, played an important role in maintaining genotypic variation within populations. Recombination and genetic differentiation in *P. violaceum*, as well as the potential for metapopulation structure, suggest the need to release additional, genetically diverse strains of the biocontrol agent at numerous sites across the distribution of the Australian blackberry infestation for maximum establishment and persistence.**

Investigating how pathogen populations evolve can provide a deeper understanding of the factors that contribute to temporal and spatial variation in disease intensity (28). This information is particularly relevant in biological control programs for weeds, when an uncharacterized population or a mixture of strains of a pathogen from the native range of the target weed has been released in the area into which the weed has been introduced. Identifying factors contributing to pathogen evolution can provide insight into how biological control can be improved through the search for and selection and release of prospective biological control agents.

There are at least 15 taxa of European blackberry (*Rubus fruticosus* L. aggregate) in Australia, where this plant is regarded as an invasive species, infesting nearly 9 million hectares of land (17, 41). Taxa of the *R. fruticosus* agg. are perennial thorny shrubs that are facultatively apomictic and genetically and morphologically variable (34). The distributions of various taxa of the *R. fruticosus* agg. can be found in a recent taxonomic update on exotic *Rubus* in Australia (17). Blackberry infestations in Australia may be discontinuous or continuous and of various sizes and shapes. More specifically, infestations can resemble linear corridors (e.g., along roadsides and in riparian zones), discontinuous patches (e.g., in invaded pastures), or continuous, blanket growth occupying vast areas such as entire valleys. In Australia, the blackberry is semidormant during winter, with active growth during spring.

Phragmidium violaceum (Schultz) Winter, a member of the Uredinales, is a fungal pathogen that causes leaf rust on the European blackberry. The pathogen is a macrocyclic, autoecious rust fungus, producing five different spore states representing the asexual and sexual components of the life cycle exclusively on taxa belonging to the *R. fruticosus* agg. The infection efficiency of this obligate biotroph is greatest on young, actively growing leaf tissue. The defoliation of the blackberry plant by rust disease results from multiple, clonal generations of airborne, dikaryotic urediniospores released during spring and summer. Teliospores represent the overwintering stage and mature on leaves that have not senesced in winter (13). As spring approaches, teliospores undergo meiosis to produce haploid basidiospores. The development of dikaryotic aeciospores and the subsequent development of urediniospores are initiated once sexual outcrossing takes place, which follows the insect-mediated transfer of spermatia (37) from spermogonia of different mating types to receptive hyphae (11). This mixed-mode reproductive strategy allows the emergence of new genotypes by recombination, while repeated clonal reproduction of wind-dispersed urediniospores facilitates the proliferation and migration of fit genotypes among

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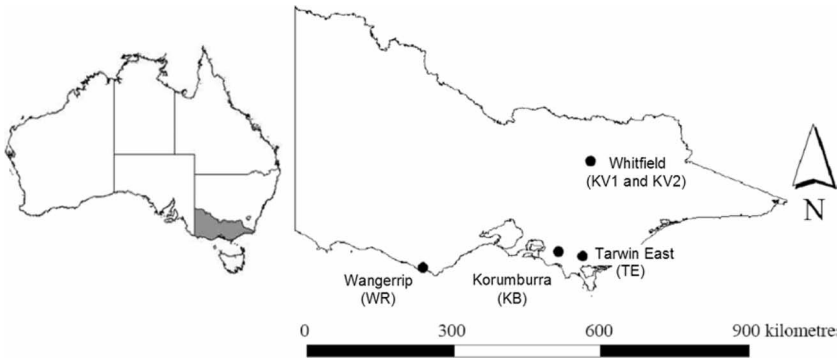

FIG. 1. Locations of populations sampled in the state of Victoria (shaded) in 2002 (KV1) and 2003 (KV2, WR, KB, and TE). The scale relates to distance on the enlarged map of Victoria displaying the locations of the populations.

susceptible hosts (28). All five spore states of *P. violaceum* are present in Australia (44), but the relative contributions of clonality, sexuality, and migration to the population structure of *P. violaceum* are unknown.

The interaction between the blackberry and *P. violaceum* appears to follow the gene-for-gene model (19), based on the results of pathogenicity studies (14). The pathogen was first reported in Australia in 1984, after an unauthorized introduction into the state of Victoria (26). This founding population of *P. violaceum* soon became widespread across blackberry infestations in temperate southern Australia (26). Subsequently, there were authorized introductions of nine strains of *P. violaceum*, collected in France, to enhance the potential of this fungus to control the European blackberry. Strain F15 of *P. violaceum* was released in 1991 and 1992, in all southern states of mainland Australia (6). The greatest number of release sites, more than 50, were in the state of Victoria (E. Bruzzese, personal communication). Eight other strains were released and strain F15 was rereleased in 2004 at experimental sites in New South Wales and Western Australia (32). The release program has since expanded and is continuing in all southern states of Australia.

We have observed marked asynchrony in disease development and severity among sites and within sites over different years. Prior to 2004, we observed some patches of European blackberry that had escaped severe disease at sites where favorable environmental conditions (high relative humidity) prevailed in summer and where there had been a significant reduction in the biomass of blackberry plants due to rust disease. Further investigations revealed fine-scale variation in the susceptibility of European blackberry plants to rust, with different clones of the same taxon collected in Australia expressing either resistance or susceptibility to a strain of *P. violaceum* (16). While it appears from the results of subsequent work that strains of *P. violaceum* that are virulent on a wide range of European blackberry taxa are present at some locations (14), the movement and introgression of virulence and resistance genes within and among populations of the pathogen and host, respectively, are undefined. Gene flow and recombination are two evolutionary forces likely to influence the population structure and evolutionary potential of *P. violaceum*, given the possibility for long-distance aerial transport of urediniospores (4) and the presence of spore states associated with sexual reproduction (44).

There is increasing evidence that biotrophic rust fungi, coevolving with their host in natural ecosystems, may exist as part of expansive genetic neighborhoods (7), known as metapopulations (24). Moreover, the geographic mosaic theory of coevolution (40) often applies to evolutionary processes of host-parasite interactions (3, 8, 9, 42, 43) because geographic differences in the fitness of members of one species are often dependent on the genetic structure (distribution of genotypes) in another species. That is, spatial heterogeneity in environmental conditions and habitat/host quality may be important in determining the genetic structure and pathogenic variation across the host-pathogen metapopulation. With these concepts in mind, DNA markers for the selective amplification of microsatellite polymorphic loci (SAMPL), described previously (21), were applied to investigate the population dynamics of *P. violaceum*.

The aim of this study was to characterize genetic diversity within and among populations of *P. violaceum* as a basis for inferring the potential for pathogen migration (gene flow) and sexual recombination. Gene flow is of particular relevance to biological control when specific strains of an agent, acting as sources for novel genes that confer virulence and/or fitness, are released for the biological control of an invasive species. While the production of wind-borne urediniospores represents an important dispersal strategy for rust fungi, it is not clear if this process translates into the frequent migration of released strains among sympatric populations of *P. violaceum*. We provide evidence that populations of *P. violaceum* in Victoria have a genetic structure that may be influenced by stochastic evolutionary processes. The potential consequences of a spatially fractured pathogen habitat and the random nature of pathogen strain recruitment are discussed in relation to the deployment of the recently introduced strains of *P. violaceum* to increase the effectiveness of the biological control of the European blackberry in Australia.

MATERIALS AND METHODS

Description of populations. An individual specimen of *P. violaceum* was defined as the urediniospores derived and propagated from a single uredinium. The individuals present at each site on a particular date are referred to, collectively,

as a population. Individual specimens of *P. violaceum* were isolated from infected leaves of European blackberry plants collected from four naturalized infestations in Victoria (Fig. 1). Samples from the Whitfield site (discontinuous infestation), located at −36.78, 146.39 decimal degrees (dd) on abandoned pastureland, were collected in November of 2002 and 2003 (populations KV1 and KV2, respectively), whereas samples from the other three sites, Wangerrip (−38.73, 143.31 dd [roadside corridor infestation]; population WR), Korumburra (−38.43, 145.80 dd [roadside corridor infestation]; population KB), and Tarwin East (−38.52, 146.24 dd [discontinous, pastureland infestation]; population TE), were collected in November 2003 only. The Korumburra and Tarwin East infestations were in the same geographical area as the location of the first discovery of *P. violaceum* in Victoria (26). The underlying host at Korumburra was predominantly *R. vestitus*, whereas *R. anglocandicans* was the predominant taxon at the remaining field sites. Both taxa belong to the *R. fruticosus* agg.

Population sampling. In order to minimize the overrepresentation of clonal genotypes, sampling was conducted during the first or second generation of uredinia, when disease intensity was low. In November 2002, population KV1 at Whitfield was sampled using a hierarchical sampling strategy for investigating genetic structure within a population (28). Samples were obtained from seven locations approximately 5 to 10 m apart along transects selected at random within the European blackberry infestation at the site. At each location, one compound leaf with uredinia from each of four primocanes originating from the same blackberry crown was sampled. However, determining the primocane origin in a dense thicket of entangled prickly shoots is very difficult, and a neighboring crown may have been sampled. Individual leaflets were separated from the compound leaf and placed in 10-cm-diameter and 2-cm-deep petri plates containing 20 ml of sterile 1% water agar (Difco Bitek agar; Becton Dickson) for transport from the field to the laboratory.

Single-uredinium-derived isolates were obtained by methods described previously by Evans et al. (15). Briefly, urediniospores from one uredinium on each of three leaflets per compound leaf were transferred, using a fine paint brush, to individual healthy leaflets of *R. anglocandicans*. Following incubation for 2 weeks, a second round of isolations was performed, single-uredinium-derived isolates were allowed to multiply, and this second generation of urediniospores was used for genetic analysis. This method of employing two rounds of single-uredinium isolations was performed to minimize contamination by nontarget DNA. Thirty-eight individuals were ultimately recovered from the 86 attempted isolations.

In 2003, 30 diseased compound leaves from different locations within each site in Victoria were sampled at random (Fig. 1). While the shape of the infestation varied among sites, sampling was performed on the equivalent areas of blackberry growth at the various sites. Detached leaflets were stored and transported as described above. To maximize the recovery of individuals, urediniospores from three randomly selected uredinia per leaf were transferred aseptically as mixed inocula onto individual healthy leaflets of *R. anglocandicans* for the isolation of single uredinia and the multiplication of urediniospores as described above. By using this method, a rate of successful recovery of individuals of between 67 and 77% was achieved from the 30 isolations that were attempted.

Analysis of genetic diversity, genetic differentiation, and potential gene flow. DNA was extracted from urediniospores of each individual, and SAMPL fingerprints were generated by following procedures described by Gomez et al. (21). Primer pairs $(GACA)_4$/H-G and R1/H-G were used to generate SAMPL fingerprints for all individuals. The presence or absence of 51 polymorphic, unambiguous, and reproducible loci (20) was scored for each individual, and the use of autoradiography prevented the scoring of low-intensity bands that might have represented contaminant nontarget DNA. The combined binary data from the 51 loci were used to determine the multilocus genotype of each individual for population genetic analyses. The genetic diversity of each population was assessed using Nei's gene diversity index (33) and Shannon's information index (39). Diversity indices were calculated using POPGENE software version 1.32 for Windows (48) and two-tailed *t* tests (50 df) performed to test for significant differences between the levels of gene diversity of any two populations.

Shannon's information index is sensitive to changes in the frequency of rare species, which may confound comparisons between populations comprising different sample sizes (35). To test if sample size might cause significant bias in the calculation of Shannon's indices for population KV1 ($n = 38$) and the other populations ($n = 20$ to 23), a bootstrap analysis was performed by calculating Shannon's information indices for 1,000 permutations of 20 randomly selected individuals from population KV1. Indices were calculated using POPGENE, and two-tailed *t* tests (50 df) were performed to test for significant differences between population KV1 ($n = 38$) and each permutation of 20 randomly sampled individuals across the 51 scored loci.

Analysis of molecular variance (AMOVA) (18) was performed using Arlequin version 2.001 for Windows (38) to assess the spatial distribution of genetic variation, from which the likely contribution of gene flow within and among populations can be inferred. AMOVA estimates variance components for SAMPL phenotypes and partitions genotypic variation within and that among populations based on the proportion of difference in the number of variable loci between all pairs of SAMPL fingerprints. The parameters estimated by AMOVA, termed Φ statistics (18), are analogous to Wright's *F*-statistics (47) and describe the level of population differentiation. The degree of differentiation between each pair of populations was also measured by calculating pairwise Φ statistics and Wright's Fst values with Arlequin and Tools for Population Genetic Analysis (TFPGA) version 1.3 for Windows (30), respectively. The calculation of Wright's Fst was based on the algorithm adjusted for data generated from dominant genetic markers (25). Under the null hypothesis of no genetic differentiation, the level of migration is expected to be high because differentiation is inversely proportional to gene flow. Genetic isolation by distance was investigated by plotting pairwise Fst and Φ values against the respective geographic distances. A regression analysis was used to test the statistical significance of the effect of geographic distance on genetic differentiation between populations (Fst and Φ).

The genetic relatedness among all SAMPL phenotypes (from 2002 and 2003) was also determined using multivariate statistics. Genetic relatedness was visualized using nonmetric multidimensional scaling (NMDS), an ordination analysis that is useful for recovering nonhierarchical patterns of genetic variation (23). NMDS is particularly effective for working with species characterized by cryptic population dynamics because of the probability of reticular evolution (46). The stress value associated with NMDS indicates how well the distribution of points on the plot matches the actual distances between observations (individuals). Stress values of <0.2 correspond to meaningful representation of the data with little chance of misrepresentation, whereas values of >0.3 generally indicate poor representation and suggest that care should be taken in interpreting the ordination (10). NMDS plots were generated from Euclidian distance measures in pairwise comparisons of unique SAMPL phenotypes identified from each population. Like the analysis of variance, the analysis of similarity (ANOSIM), based on Euclidian distance, was used to assess the congruence among individuals grouped according to their respective populations. Under the null hypothesis of no differentiation, the test statistic *R* changes little when labels identifying populations are rearranged randomly. *R* values range between 1 (maximum separation) and 0 (no separation) (10). The associated measure of significance, *P*, was calculated over 1,000 permutations. NMDS and ANOSIM were performed using the multivariate statistical software package Primer version 6 (PRIMER-E Ltd., Plymouth Marine Laboratory, Plymouth, United Kingdom).

Evidence for recombination. To test for random mating, multilocus linkage disequilibrium was measured by calculating the index of association (I_A) (27) using the Multilocus software (1). The I_A observed for each population was compared with the I_A expected in the context of random mating simulated through the reshuffling of clone-corrected data over 1,000 permutations.

TABLE 1. Number of unique individuals and diversity indices, based on 51 SAMPL loci, observed for populations of *P. violaceum* sampled in Victoria in 2002 and 2003

Population[a]	No. of samples	No. of SAMPL fingerprints identified	Nei's gene diversity index	Shannon's information index
KV1[b]	38	29	0.10	0.14
KV2[c]	21	19	0.15	0.21
TE[c]	23	21	0.12	0.17
WR[c]	23	21	0.09	0.13
KB[c]	20	2	0.04	0.05

[a] Refer to Fig. 1 for population locations.
[b] Population sampled in 2002.
[c] Population sampled in 2003.

RESULTS

Analysis of genetic diversity, genetic differentiation, and potential gene flow. In 2002, 29 SAMPL phenotypes were identified among 38 individuals collected from population KV1 at Whitfield (Table 1). AMOVA revealed that there was no sig-

TABLE 2. AMOVA (18) among 38 individuals of the species *P. violaceum*, population KV1, collected from seven locations within a single field site at Whitfield, Victoria, in 2002, based on 51 SAMPL loci

Comparison	df	Sum of squares	Variance component	% Variation	Φ[a]	*P*
Among locations within the field	6	12.46	0.07	4.17	0.04	0.14
Within locations	31	52.17	1.68	95.83		

[a] Φ is a statistic analogous to Wright's Fst values (18).

nificant genetic differentiation among the specimens from the seven locations within this single site ($\Phi = 0.0417$; $P = 0.140$) (Table 2), indicating that the recovered individuals likely belonged to the one population. Based on this result, individuals were sampled at random across locations within each infestation for all populations studied in 2003.

In 2003, 20 to 23 individuals from a potential maximum of 30 were recovered from each field site, giving a total of 87 individuals for population genetic analysis. SAMPL revealed considerable genotypic variation in populations KV2, TE, and WR, with 60 of the 67 individuals in these three populations representing unique SAMPL fingerprints (Table 1). In contrast, 19 of the 20 individuals from population KB yielded a single clone. In total, 61 unique SAMPL fingerprints were identified among the 87 isolates collected in 2003. Populations KV2 and WR shared one clone, as did populations KB and TE. These shared clones were detected once in each population. The clone shared by populations KB and TE corresponded to the genotype detected once in the KB population.

Population KV1, obtained in 2002, did not share any SAMPL fingerprints with population KV2, which was obtained from the same weed infestation in the following growing season. Bootstrap analysis revealed that 99.7% of Shannon's information indices calculated for 1,000 permutations of 20 randomly selected individuals from population KV1 were not significantly different ($P > 0.05$; *t* test) from those calculated for the original population (KV1; $n = 38$). This result indicated that meaningful comparisons between population KV1 and the populations sampled in 2003 could be made. No significant difference ($P > 0.05$; *t* test) between genetic diversity indices was observed in pairwise comparisons, with the exception that population KB was significantly less diverse than populations TE, KV1, and KV2 ($P < 0.05$; *t* test), according to both Nei's gene diversity indices and Shannon's information indices.

AMOVA revealed that 59.9% of the variation occurred within populations and 40.1% was observed among populations in 2003 (Table 3). This result suggests significant structure or differentiation among the populations ($\Phi = 0.4019$; $P < 0.0001$), implying that migration was infrequent among these demes. Φ and Fst values for individual pairwise comparisons between populations collected in 2002 (KV1) and 2003 revealed significant differentiation between all pairs of populations ($P < 0.0001$) (Table 4), with Φ values ranging from 0.13 to 0.74. The variation in the relative level of genetic separation indicated that rates of migration varied among different populations. No apparent association between the degree of population differentiation (Φ and Fst) and geographic distance was observed (Fig. 2). A regression analysis supported this lack of association, with geographic distance having no significant effect on Φ and Fst ($P = 0.658$ and 0.825, respectively).

TABLE 3. AMOVA (18) among 87 individuals of the species *P. violaceum* collected from four populations in Victoria in 2003, based on 51 SAMPL loci

Comparison	df	Sum of squares	Variance component	% Variation	Φ[a]	*P*
Among populations	3	99.43	1.43	40.1	0.4019	<0.0001
Within populations	83	176.92	2.13	59.9		

[a] Φ is a statistic analogous to Wright's Fst values (18).

TABLE 4. ANOSIM *R* or Φ values for pairwise comparisons between populations[a]

Population	Value for comparison with:				
	WR	TE	KB	KV2	KV1
WR		0.21	0.99	0.41	0.91
TE	0.17		0.77	0.19	0.77
KB	0.74	0.58		0.53	0.93
KV2	0.28	0.13	0.35		0.65
KV1	0.49	0.40	0.57	0.28	

[a] Refer to Fig. 1 for population locations. Values in the upper right are *R* values (10), and those in the lower left are Φ values (18). A significant difference ($P < 0.001$) was observed for each pairwise comparison. Differentiation increases with increasing values of *R* and Φ.

The application of NMDS had an associated two-dimensional-stress level of 0.16 (Fig. 3), indicating that the NMDS plot generated was an appropriate representation of the data. SAMPL fingerprints from population KV1 and six fingerprints from population KV2 formed one cluster distinct from another cluster composed of the fingerprints of individuals from the remaining populations. SAMPL phenotypes from populations KV1 and WR formed tight clusters, indicating high levels of genetic similarity among strains in these populations. ANOSIM supported observations from AMOVA with a global *R* statistic of 0.676 ($P < 0.001$), indicating clear separation among populations. Likewise, *R* values for pairwise compari-

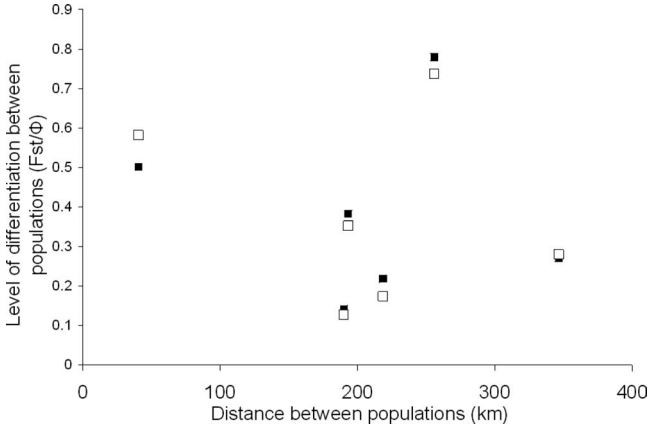

FIG. 2. Relationship between the level of genetic differentiation of the 2003 *P. violaceum* populations, expressed as Φ (□) or Fst (■), and geographic distance. A regression analysis indicated no significant effect of geographic distance on Φ and Fst ($P = 0.658$ and 0.825, respectively).

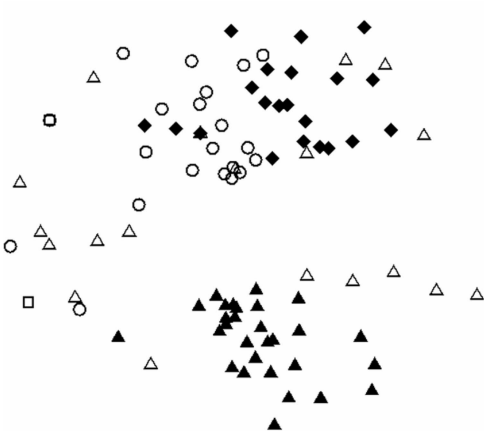

FIG. 3. Genetic relationship among unique SAMPL phenotypes recovered from populations KV1 (▲), KV2 (△), WR (◆), TE (○), and KB (□) as visualized by an NMDS plot with a two-dimensional-stress level of 0.16. Individuals from populations KV1 and WR were found to have a random association of loci based on the calculation of I_A, an indication that these populations were panmictic.

sons revealed a significant difference between any two populations ($P < 0.001$) (Table 4).

Evidence for random mating. The KB population was omitted from I_A analyses as only two clones were identified among the 20 individuals collected. Evidence of panmixia (the random association of loci) was supported for populations KV1 and WR, with I_A values of −0.101 ($P = 0.83$) and −0.088 ($P = 0.72$), respectively. Populations TE and KV2 displayed significant nonrandom associations among their fingerprints, with I_A values of 0.413 ($P = 0.02$) and 1.117 ($P < 0.01$), respectively. Therefore, the hypothesis of random mating within these two populations was rejected.

DISCUSSION

Population dynamics of *P. violaceum*. In the 7 years between the first report of *P. violaceum* in Australia as localized disease foci in 1984 and the release of strain F15 for biological control in 1991, *P. violaceum* became widespread on European blackberry infestations across Australia (26, 45). Despite the potential for long-distance dispersal of urediniospores (4), the populations of *P. violaceum* studied appeared to be evolving in relative isolation, based on the observation of significant genetic differentiation among populations and a lack of association between genetic and geographic distance. Milgroom and Lipari (29) quantified a similar phenomenon among highly differentiated populations of the ascomycete *Cryphonectria parasitica* (chestnut blight fungus), which also has spores (ascospores) that are dispersed long distances by the wind. Variable weather patterns may transport spores in any direction, even though the migration of urediniospores is likely to be in the direction of prevailing winds (2). While the potential distance of pathogen migration is large, heterogeneity in environmental conditions and habitat quality, characterized by a genetically variable host, may limit the establishment of migrating individuals. Under these circumstances, stochastic processes such as random genetic drift and random migration are likely to play important roles in maintaining genetic differentiation among pathogen demes (7). Nevertheless, information on how alleles recombine in *P. violaceum* is required before migration can be confirmed as a dominant factor in the genetic differentiation of populations, given the assumptions underlying the calculation of the genetic statistics. This information, combined with more intensive sampling within and among populations of *P. violaceum* in Victoria, is needed before conclusions about metapopulation structure can be made.

Sexual recombination was found to be an important process driving change within populations of *P. violaceum*, with high levels of genotypic variation observed in three of four populations and the statistical determination of the occurrence of random mating in two populations (KV1 and WR). The association of temporal variation with panmixia and genetic structure was supported for one site (Whitfield; populations KV1 and KV2); however, further work is required to investigate if this temporal variation is random for all populations of *P. violaceum*. The spatial variation in the occurrence of random mating among populations in 2003 may be due to the stochastic nature of migration. If pathogen movement is reticular and random, the spatial definition of a population may not be consistent for all demes. That is, the degree of divergence (genetic isolation) among populations may fluctuate over time, depending on the available sources of migrants. Furthermore, sources and sinks of migrating individuals may also fluctuate due to the asynchronous nature of disease development across fragmented habitats. If so, genetic diversity may be underestimated through sampling error, and statistical analyses such as I_A may be skewed.

The population from Korumburra (KB) had an obvious clonal structure in 2003. Although *R. vestitus* was the predominant taxon at Korumburra, it is not known whether the host was genetically variable or whether it had a complex genetic structure for resistance to *P. violaceum*. Thus, it is not clear if host-mediated selection pressures contributed to the selection and proliferation of a fit, virulent pathogen genotype at this site. Alternatively, the lack of genotypic diversity in *P. violaceum* at this site may have been a result of a genetic bottleneck followed by the proliferation of the resulting pathogen genotype(s) over several generations before sampling, with little or no immigration of unique genotypes from other populations. Weather conditions are often conducive for multiple cycles of infection by *P. violaceum* in this region (36), which would have allowed for the proliferation of the dominant genotype on susceptible plants in the infestation.

The observation of a genotype in population KB that was also observed in population TE may have arisen through the contamination of the KB samples or sampling errors in the laboratory. However, individuals from populations were propagated on detached leaves at different times, and urediniospores from each population were stored separately to minimize the chance of error. Although the mechanisms contributing to the genetic structure at this site are not clear, the observations provide support for the involvement of stochastic processes in the evolution of pathogen populations across habitats (and host populations) that are spatially fragmented.

Population dynamics in relation to biological control. Where recombination is important for maintaining and generating genotypic variation, as shown here for *P. violaceum*, nat-

ural selection favors virulent genotypes of the pathogen that are able to establish disease on susceptible hosts. Recent work in the Australian biological control program for the European blackberry was initiated on the basis of the findings reported here and in the study by Gomez et al. (21). The program has focused on the selection and release of additional strains of *P. violaceum*, potentially possessing a diverse array of alleles associated with virulence and fitness, to provide increased opportunity for the fungus to evolve and adapt to its environment through natural selection (31, 32). This strategy was not employed at the commencement of the biological control program in Australia, when only strain F15 was released because it appeared to cause significant disease in common taxa of the *R. fruticosus* agg. in Victoria (5).

The results of this study highlight some of the potential pitfalls in releasing pathogen strains for the biological control of a genetically diverse weed when the release is done in an ad hoc or restricted manner, as influenced by regulation, funding, and/or unauthorized incursions of the pathogen. While there are general rules that predict the success of the establishment of an exotic organism (12), the amount of effort expended to introduce a biological control agent is likely to be the key to successful establishment (22). However, there is a high potential for the failure of additional strains of *P. violaceum* because of the "numbers game" involved in pathogen strain recruitment. That is, the amount of inoculum of a single strain of *P. violaceum* upon initial release is small compared with the well-adapted existing pathogen population. Consequently, there is a high risk that a released clonal genotype may soon become extinct, unless it recombines and its genes are introgressed into the existing population soon after establishment. There is evidence to suggest that strain F15 of *P. violaceum* did not persist in the Australian environment, but whether or not genes originating from this strain are present in the current population of *P. violaceum* is unknown (15, 21).

The potential for *P. violaceum* to have a metapopulation structure suggests that the release of a "super fit" strain (or individual), an approach commonly used in biological control, would be inappropriate. When a rust strain becomes established at the site where it is released, there is no guarantee, according to metapopulation theory, that the genotype or genes will disperse to another location and become well established there, despite the apparent capacity for long-distance dispersal (4). Genetic differentiation and recombination in *P. violaceum* suggest the need to release multiple rust strains at numerous sites across the distribution of the European blackberry and over several years to accommodate the temporal and spatial asynchrony in pathogen and disease dynamics. In short, the data presented here highlight the need for a significant change in selection and release strategies for sexually recombining rust fungi used for the biological control of genetically diverse weeds.

Monitoring the fate and efficacy of strains of a pathogen released in a new environment is the final phase of a biological control program, although this important phase has been omitted in some programs when resources were limited. Even though SAMPL markers resolve considerable genetic diversity in *P. violaceum* (21), the dominant nature of these markers makes it difficult to determine heterozygosity and to track strains (and their genes) in panmictic populations. The markers must also be accompanied by rigorous procedures to minimize the amplification and scoring of loci from nontarget DNA. The development of polymorphic, codominant, single-locus, and species-specific microsatellite markers is under way and should improve the efficiency and accuracy of postrelease monitoring of the additional strains introduced in 2004. These strains are currently being released on a large scale across temperate southern Australia. Analyses of *P. violaceum* populations after these releases will offer the opportunity to test hypotheses about increasing genetic diversity in *P. violaceum* for postrelease adaptation and improved biological control. The concurrent application of genetic markers for the *R. fruticosus* agg. would also enable the study of coevolution in the blackberry-rust pathosystem.

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